March 30, 2014

Ms. Monet Vela
Office of Environmental Health Hazard Assessment
P.O. Box 4010, MS-19B
Sacramento, California 95812-4010

RE: Notice of Intent to List – Styrene; Health and Safety Code Section 25249.8(B) and Title 27, Cal. Code of Regs., Section 25902; February 27, 2014.

Dear Ms. Vela:

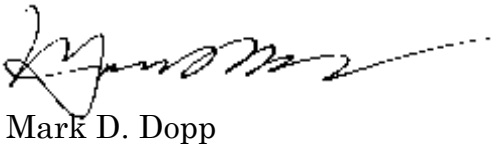
The North American Meat Institute (NAMI or the Meat Institute) submits this letter in response to an invitation for comments in the above-referenced notice of intent to list published by the California Environmental Protection Agency's Office of Environmental Health Hazard Assessment (OEHHA). NAMI is the nation's oldest and largest trade association representing packers and processors of beef, pork, lamb, veal, turkey, and processed meat products. Meat Institute member companies account for more than 95 percent of United States output of these products and more than 100 NAMI members operate in California. Some NAMI members produce meat and poultry products that utilize packaging material of which styrene is a component and for that reason NAMI has a direct interest in the published notice.

The Meat Institute has reviewed the information provided by OEHHA and the comments submitted on March 26, 2015, by the Styrene Research and Information Center (SIRC).[1] NAMI concurs with SIRC's comments and adopts them *en toto* for this submission.

[1] See Attachment A.

If you have questions regarding any aspect of these comments or would like to discuss them please contact me.

Respectfully submitted,



Mark D. Dopp
Senior Vice President, Regulatory and
Scientific Affairs, & General Counsel

Attachment

Attachment A

COMMENTS OF THE

STYRENE INFORMATION RESEARCH CENTER

ON

NOTICE OF INTENT TO LIST STYRENE

UNDER THE AUTHORITATIVE BODIES LISTING MECHANISM

HEALTH AND SAFETY CODE SECTION 25249.8(B) AND

TITLE 27, CAL. CODE OF REGS., SECTION 25902

MARCH 26, 2015

I. Introduction and Summary

On February 27, 2015, the California Environmental Protection Agency's Office of Environmental Health Hazard Assessment (OEHHA) published a notice of intent to list styrene as known to the State to cause cancer under the Safe Drinking Water and Toxic Enforcement Act of 1986.[1] This action was taken under the authoritative bodies listing mechanism.[2]

OEHHA based its notice on a June 2011 listing by the National Toxicology Program (NTP) of styrene as "Reasonably Anticipated to be a Human Carcinogen" in the Report on Carcinogens, Twelfth Edition (RoC). In the final substance profile, NTP explained the basis for listing as follows:

> Styrene is reasonably anticipated to be a human carcinogen based on limited evidence of carcinogenicity from studies in humans, sufficient evidence of carcinogenicity from studies in experimental animals, and supporting data on mechanisms of carcinogenesis.

Proposition 65 requires that there be sufficient evidence in humans, or sufficient evidence in animals supported by additional evidence demonstrating the relevance of the animal data to human carcinogenicity. Authoritative bodies listing proposals require OEHHA to consider new scientific data and data not considered by NTP. A listing may not proceed if it is established that the sufficiency of evidence criteria were not met.

- After briefly comparing the NTP classification and Proposition 65 listing criteria, in section III, we present the data and their regulatory significance in its traditional sequence of human, animal, and other data. This discussion supports three interrelated conclusions. First, new human studies published after the RoC listing demonstrate that the human evidence is inadequate, and not limited. Second, because NTP did not or was unable to consider scientifically valid data, the animal evidence is not sufficient, precluding a listing. Third, additional mode of action data demonstrate that the animal data are not relevant to humans, and styrene does not present a human cancer risk at anticipated exposure levels.

- The NTP listing concluded that the evidence in humans was limited. Therefore, the requirement of sufficient evidence in humans is not met by the listing of styrene in the RoC. In its Public Health Goal document for styrene, OEHHA also concluded that the human data are limited. Based upon recent updates to the major epidemiology studies of

[1] Commonly known as Proposition 65, the Safe Drinking Water and Toxic Enforcement Act of 1986 is codified in Health and Safety Code § 25249.5 et seq.

[2] *See* Health and Safety Code § 25249.8(b) and Title 27, Cal. Code of Regs. § 25902 ("Formally Required to Be Labeled or Identified").

reinforced plastics workers exposed to styrene which refute earlier proposed increases in cancer of the hematopoietic and lymphatic systems, the requirement of sufficient evidence in humans is not satisfied. Therefore, the data from human studies do not support a listing of styrene under Proposition 65.

- The styrene data in animals were described as sufficient in the RoC, based on increased lung tumors in mice by two routes of exposure – inhalation and oral. This conclusion is based on increased lung tumors in male and female mice exposed by inhalation (Cruzan 2001) and increased lung tumors in male mice from gavage administration of styrene (NCI 1979). In the NCI (1979) gavage study, the incidence of lung tumors in the treated mice were within the historical control range of mice tested at the same laboratory at about the same time; the original report concluded there was no more than suggestive evidence of increased tumors. In 2002, the International Agency for Research on Cancer (IARC) concluded the animal data were limited, not sufficient. Uncharacteristic of its established practice, NTP ignored the conclusions of the authors of the NCI 1979 study, ignored IARC's contrary evaluation, ignored previously published studies and departed from accepted science by using historical controls from another laboratory in its analysis of the oral gavage study in mice. NTP's inclusion of external historical controls led to the conclusion that sufficient evidence existed in mice following oral administration. Because NTP's misuse of external historical controls was inappropriate, NTP's sufficient evidence conclusion cannot be considered by OEHHA. Thus, the animal evidence is limited, and not sufficient. No authoritative bodies listing may be made.

- Mode of Action (MOA) research, including three later studies not considered by NTP, indicates that the mouse lung tumors are caused by mouse-specific metabolism by the enzyme CYP2F2, which does not occur in rats or humans. Thus, the animal data are irrelevant to human cancer risk.

Based on new human and mode of action studies, coupled with NTP's failure to consider scientific data and well-established scientific principles in reinterpreting NCI (1979), there is no basis for OEHHA to list styrene under the authoritative bodies listing mechanism because it is clearly established that the sufficiency of evidence criteria were not met.

II. Comparison of Proposition 65 and NTP Listing Criteria

OEHHA's authoritative body notice of intent to list is governed by § 25306 (Chemicals Formally Identified by Authoritative Bodies) in Title 27, Division 4, Chapter 1 of the California Code of Regulations. Key regulatory criteria for listing appear in the definition of causing cancer, and what additional data or factors must be considered by OEHHA in determining whether to list.

The definition for substances that may be listed under the authoritative bodies mechanism is found in § 25306(e) (Cancer Defined), which states:

> (e) For purposes of this section, "as causing cancer" means that either of the following criteria has been satisfied:
>
> > (1) sufficient evidence of carcinogenicity exists from studies in humans. For purposes of this paragraph, "sufficient evidence" means studies in humans indicate that there is a causal relationship between the chemical and cancer.
> >
> > (2) sufficient evidence of carcinogenicity exists from studies in experimental animals. For purposes of this paragraph, "sufficient evidence" means studies in experimental animals indicate that there is an increased incidence of malignant tumors or combined malignant and benign tumors in multiple species or strains, in multiple experiments (*e.g.*, with different routes of administration or using different dose levels), or, to an unusual degree, in a single experiment with regard to high incidence, site or type of tumor, or age at onset.

In addition to the threshold criteria in the definitional section, § 25306(f) describes the additional reasons that OEHHA should not list. Subsection (f) provides:

> (f) The lead agency shall find that a chemical does not satisfy the definition of "as causing cancer" if scientifically valid data which were not considered by the authoritative body clearly establish that the chemical does not satisfy the criteria of subsection (e), paragraph (1) or subsection (e), paragraph (2).

Both of the definitional provisions expressly incorporate the sufficient evidence requirement, which includes the question of whether the listing of styrene in the Report of Carcinogens is consistent with accepted scientific principles. The reasons-not-to-list provision covers two sets of data with respect to the NTP's 2011 listing, including both: (1) any information developed after the June 2011 listing, and (2) any data not considered by NTP during the listing process.

Additionally, when promulgating § 23506 in February 1990, the California Health and Welfare Agency explained that, in evaluating whether an authoritative body had formally identified chemicals as causing cancer or reproductive toxicity, the agency would determine whether the authoritative body had relied on satisfactory human or animal studies. The agency explained that:

> Where there is in fact an insufficient number of positive or studies, but the authoritative body has concluded anyway that the chemical causes cancer, the Agency will be prevented by the regulation from bringing the chemical to the list. The Agency will not completely defer to the authoritative body, and will at least determine that the body relied upon the requisite human or animal studies.

Thus, even if an authoritative body, such as NTP, has decided to identify a chemical as causing cancer or reproductive toxicity, its conclusion must be investigated for the sufficiency – including an adequate number of human or animal studies – before OEHHA decides that a substance may be listed.

III. The NTP Report on Carcinogen Listing Does Not Support Listing under Proposition 65

A. Human Data

NTP's RoC found the human data on the carcinogenicity of styrene to be limited. This does not support listing under Proposition 65.[3] Further, the RoC's evaluation was based on studies that have since been updated. These data were not available to NTP and so, reasonably, they were not considered. These new data demonstrate, however, the inadequacy of the human data that NTP considered on the carcinogenicity of styrene. This includes studies of human data based on a cohort of US reinforced plastics and composite (RPC) workers by Wong et al., (1994); a cohort of Washington state RPC workers by Ruder et al., (2004); and a combination of 8 cohorts of RPC workers in the EU by Kogevinas et al., 1994). Wong and Ruder found no styrene-related increases in cancer. Kogevinas reported no styrene-related increases in cancer based on cumulative exposure or duration of exposure, but an increase in total lymphomas based on average exposure. Part of the Kogevinas cohort was taken from a cohort of Danish workers that may have been exposed to styrene in RPC operations (Kolstad et al, 1994). Workers were divided based on companies where less than 50% of the workers were thought to be involved in RPC operations and companies where more than 50% were thought to be involved. Kolstad reported increased leukemia in the overall cohort among workers hired before 1960 and among those employed for less than 1 year. Workers from companies where more than 50% were thought to have been involved in RPC operations were included in the Kogevinas study.

After the NTP concluded its review, the Wong, Kogevinas, Ruder, and Kolstad studies have been updated. Each update shows a lack of styrene carcinogenicity, vitiating prior assessments to the contrary.

- Collins et al., (2013) updated the Wong cohort of US RPC workers. More than 85% of the cohort has been followed for more than 30 years. There were no increased incidences of leukemias or lymphomas based on cumulative exposure, average exposure, duration of exposure or peak exposures. Increased lung cancer followed an inverted dose-response pattern; *i.e.*, lowest incidence among highest exposed workers, and was attributed to smoking.

[3] *Styrene Information Research Center v. Office of Environmental Health Hazard Assessment*, 210 Cal. App. 4th 1082, 1101 (2012).

- Coggon et al., (2014) have updated their cohort of the Kogevinas study. They found no styrene-related increase in cancer.

- Ruder et al., (2014) presented an update of Washington state RPC cohort in June 2014 at the conference "Challenges for Occupational Epidemiology in the 21st Century." No styrene-related cancers were reported.

- Kolstad et al., (2014) presented update of Danish RPC workers in June 2014 at the conference "Challenges for Occupational Epidemiology in the 21st Century." No styrene-related cancers were reported.

Collectively, these new studies demonstrate the proposition that styrene is *not* carcinogenic in humans. The prior limited evidence characterization of the human data by NTP in the RoC is, then, undermined by this human data that were not available to the NTP and thus could not have been – and was not – considered by NTP. Given the number of human studies, the number of workers followed and the length of follow up, a conclusion that styrene is not a human carcinogen is well-founded.

B. Animal Data

Based on two studies, NTP found that the data from animal studies in different strains of mice were sufficient to support its decision that styrene is reasonably anticipated to be a carcinogen. Adverse findings in both studies are essential to support a finding of sufficient evidence. In concluding that the animal data constituted sufficient evidence of carcinogenicity, however, NTP did not consider scientifically valid data. Because one of the two studies does not demonstrate an increased incidence of tumors in animals, the single remaining study that NTP cited cannot serve as the basis for listing; a single study does not constitute sufficient evidence under § 23506.

One of these studies was conducted by the National Cancer Institute (NCI) in B6C3F1 mice and reported in 1979. Styrene was administered by gavage, that is, orally by a tube inserted into the stomach. NCI concluded that the data provided only "suggestive" evidence of carcinogenicity and under the conditions of the study "no convincing evidence" of carcinogenicity of styrene was obtained. NTP, despite NCI's conclusion that the evidence was not convincing, only suggestive, arrived at a contrary and scientifically inconsistent decision by using control data that were not part of the NCI study.

Animal studies involve treated animals, that is, animals exposed to the chemical being tested. Typically, chemicals will be administered to the treated animals in two or three different amounts, low and high levels with a possible medium level. The laboratory conducting the study will also involve control animals, that is, animals living in identical circumstances with the same housing, food, water, and environmental conditions, except they will not be exposed to the chemical. The effect of the exposure in the treated animals is then compared against control

animals. This comparison is used to determine whether the treated animals show a statistically significant increase in tumors (or other possible effects being studied) at the various levels of exposure than the control animals develop.

To minimize the potential effect of unusual results in the control animals in a single study that would distort the study results, historical controls are used. Historical controls are the same species and strain of animal, used in other studies conducted by the same laboratory under comparable conditions. It is imperative that the historical controls be from the same laboratory to assure that all the controls were subject to the same circumstances. Otherwise, differences in the animals' circumstances could affect the outcomes for the controls.

Using controls from the same laboratory to constitute the historical controls is a generally accepted scientific principle. Ignoring this principle, NTP used the outcome for controls from another laboratory to calculate the number of tumors developed in the controls to compare against the number of tumors in the treated mice.

NTP selected data from 12 studies conducted at a different laboratory and only two from the laboratory where NCI conducted its gavage study. Data for controls from other studies at the laboratory conducting the NCI study were available, but not selected by NTP.

The controls at the laboratory where the NCI study was conducted developed three times more tumors than the controls at the other laboratory. The use of the control data from the other laboratory exaggerated the difference between the "controls" and the treated animals. NTP used this exaggerated difference to rationalize its conclusion that the data were sufficient.

NTP did not consider sound and objective scientific practice in using animals from a laboratory different than that used in NCI (1979a). NTP justified this action as being required to obtain a sufficient number of controls for studies that used corn oil as the vehicle for administration of the test substance. In so doing, NTP did not follow its traditional practice of not engaging in additional analyses of historical controls when it chose NCI as the sole study among the hundreds referenced in the Background Document for which it chose this unusual approach.

Specifically, NTP did not consider existing data regarding (i) the effect of a corn oil vehicle for administration of the test substance, and the (ii) appropriateness of mixing controls from different labs. As to the corn oil issue, NTP's own analysis of the NTP historical control database (Haseman et al., 1985) concluded that use of corn oil vehicle in the NCI study specifically *did not* impact lung tumor incidence in the B6C3F1 mice used in NCI-NTP carcinogenesis bioassays. Importantly, NTP did not reference Haseman et al., (1985), showing that NTP did not consider scientifically valid evidence before coming to its conclusion that there is sufficient evidence of carcinogenicity of styrene in experimental animals.

In stark contrast to its treatment of NCI 1979, NTP departed from its published position that, because of significant inter-laboratory variability in the incidence of background mouse lung tumors, historical control tumor analyses for this endpoint should be restricted to tumor incidences observed within the same testing laboratory (Haseman et al., 1984). In Keenan et al., (2009) the authors, which included representatives of NTP, NIEHS, FDA, and USEPA, recommended consensus principles to guide the use of historical control data from chronic rodent bioassays. Their first consensus principle is that the "current control group is the most relevant comparator for determining treatment-related effects in a study."

In a 2002 review of styrene, the International Agency for Research on Cancer (IARC) considered both Cruzan, et al. (2001) and NCI (1979), in addition to other animal studies. IARC (2002). NTP did not consider that IARC reached a different conclusion, and that IARC found the animal evidence to be limited. IARC's summary of NCI (1979) explicitly referred to historical controls, meaning that IARC was aware of the issue, as the last sentence of the relevant paragraph from the IARC Monograph states (emphasis added:

> Groups of 50 male and 50 female B6C3F1 mice, six weeks of age, received daily administrations of 150 or 300 mg/kg bw styrene (purity, 99.7%) in corn oil by gavage on five days per week, for 78 weeks, and the animals were killed after a further 13 weeks. Control groups of 20 male and 20 female mice received corn oil alone. The incidence of bronchiolo-alveolar carcinomas in males was 0/20, 3/44 and 5/43, while the incidence of adenomas and carcinomas combined was 0/20, 6/44 and 9/43 (p = 0.024) for doses of 0, 150 and 300 mg/kg, respectively. There were no bronchiolo-alveolar carcinomas in female mice. The incidence of bronchiolo-alveolar adenomas in females was 0/20, 1/43 and 3/43, respectively (National Cancer Institute, 1979a). [*The Working Group noted the small number of control animals and that the incidence of both adenomas and carcinomas combined was within the historical control ranges.*]

IARC (2002) demonstrates that NTP's approach was outcome determinative and that, had NTP considered the relevant data and applied the relevant science policies, NCI (1979) would not have provided the animal evidence necessary to add styrene to the Report on Carcinogens. More importantly, OEHHA "will not completely defer to the authoritative body, and will at least determine that the body relied upon the requisite human or animal studies." "Where there is in fact an insufficient number of positive studies, but the authoritative body has concluded anyway that the chemical causes cancer, the Agency will be prevented by the regulation from bringing the chemical to the list."

That is the present situation because NTP did not consider scientifically valid data with regard to the use of historical controls and the difference in the data between the external laboratory controls it used when manipulating the comparison with historical controls. The failure to

consider these data is critical since NTP's novel analysis was necessary to support its conclusion that the animal tumorigenicity data justified the proposed "reasonably anticipated as a human carcinogen" RoC listing. NTP's analysis does not provide the needed support for listing styrene under Proposition 65. Because there is only one animal study upon which OEHHA can rely, no listing of styrene is permitted. [4]

C. Mode of Action

The second animal study upon which NTP relied was conducted by Cruzan et al., in CD-1 mice and reported in 2001. These mice were exposed by inhalation and developed lung tumors. However, the tumors resulted from a mechanism of action that does not occur in humans or even in other animal species, including rats.

- NTP hypothesized that mouse lung tumors developed as a consequence of styrene metabolizing to styrene oxide. Studies, available to but not considered by NTP, demonstrate that styrene oxide does not cause lung tumors in mice or rats.
- The mechanism of action causing lung tumors is unique to mice. Styrene is metabolized in mouse lungs by an enzyme, CYP2F2, causing cytotoxicity, that is, cell damage. The resulting regeneration of cells to repair the damage causes increased cell replication (hyperplasia) and eventually lung tumors.
- Laboratories have bred mice with the CYP2F2 enzyme "knocked out." These knockout mice do not experience cytotoxicity when they are exposed to styrene.
- Rats do not have the CYP2F2 enzyme. The rat counterpart is CYP2F4. Styrene does not cause cytotoxicity or lung tumors in rats. Humans do not have the CYP2F2 enzyme. The human counterpart is CYP2F1, and it is present at a much lower level even than the CYP2F4 enzyme in rats.
- Laboratories have developed mice with the human CYP2F1 enzyme rather than the CYP2F2 mouse enzyme. These "humanized" mice do not experience cytotoxicity when exposed to styrene.

These conclusions are bolstered by four studies not considered by NTP that were published after the Report on Carcinogens. They are:

[4] In developing a new analysis or interpretation of the original study using additional data, NTP also departed from its policy stating that it only relies on peer-reviewed studies in preparing the Background Document. The new analysis should have first been published in a peer review journal. That process would have provided the necessary scientific scrutiny and comparison with consensus practices. NTP has never done this. In any event, NTP's failure to consider Haseman (1984 and 1985) and Keenan (2009) preclude the listing of styrene by OEHHA.

1. Carlson, G.P. 2012. Modification of the metabolism and toxicity of styrene and styrene oxide in hepatic cytochrome P450 reductase deficient mice and CYP2F2 deficient mice. Toxicology 294(2-3):104-108.
2. Cruzan, G; Bus, J; Hotchkiss, J; Harkema, J; Banton, M; Sarang, S. (2012). CYP2F2-generated metabolites, not styrene oxide, are a key event mediating the mode of action of styrene-induced mouse lung tumors. Regul Toxicol Pharmacol 62: 214-220. http://dx.doi.org/10.1016/j.yrtph.2011.10.007
3. Cruzan, G; Bus, J; Hotchkiss, J; Sura, R; Moore, C; Yost, G; Banton, M; Sarang, S. (2013). Studies of styrene, styrene oxide and 4-hydroxystyrene toxicity in CYP2F2 knockout and CYP2F1 humanized mice support lack of human relevance for mouse lung tumors. Regul Toxicol Pharmacol 66: 24-29. http://dx.doi.org/10.1016/j.yrtph.2013.02.008
4. Shen, S; Li, L; Ding, X; Zheng, J. (2014). Metabolism of styrene to styrene oxide and vinylphenols in cytochrome P450 2F2- and P450 2E1-knockout mouse liver and lung microsomes. Chem Res Toxicol 27: 27-33. http://dx.doi.org/10.1021/tx400305w

Further elaboration of these points follows, but because NTP did not consider these data, an authoritative bodies listing may not proceed.

1. Mode of Action of Mouse Lung Tumors Requires CYP2F2 Metabolism

Do the mouse lung tumors provide evidence of human cancer? This can be examined by determining the Mode of Action (MOA) by which styrene induces lung tumors in mice. Facets examined include toxic effects, cells affected, metabolic considerations, and gene mutations. Cruzan et al., (2015) examined mouse lung genomic responses in styrene treated wild-type CYP2F2 knockout and CYP2F1 humanized mice. The evidence supports the conclusion that the mouse-specific lung toxicity and tumorigenicity are not relevant to humans. The metabolism of styrene by mouse lung CYP2F2 to cytotoxic metabolite(s) has been postulated as an essential step for mouse lung toxicity and mouse lung specific tumorigenicity (Cruzan et al., RTP, 2012, 2013). The purpose of this study was to use whole-lung genomic analysis to further investigate potential MOAs of styrene in C57BL/6 wild-type, CYP2F2 knockout (-/-; KO) and CYP2F21 humanized (2F2-KO + 2F1,2A13,2B6-transgenic, TG) male mice. Mice were exposed to 0, 40, or 120 ppm styrene for 6 hours a day, 5 days a week, for either 1 or 4 weeks. Five biological replicates for each treatment group were analyzed for relative gene expression using Affymetrix whole genome HT_MG430_PM Titan arrays. 287 genes were significantly differentially expressed in wild-type mice at both styrene concentrations. Gene ontology enrichment showed a strong dominance of cell cycle regulatory pathways consistent with cell proliferation. No genes were significantly differentially expressed in knockout mice. Only a single gene was significantly differentially expressed at 120 ppm in transgenic mice after 1 week and a different single gene at 40 ppm after 4 weeks. This study supports the conclusion that the MOA of styrene mouse lung toxicity requires CYP2F2 metabolism (but not by human CYP2F1) as a key gateway

event, and also evidences that alternative MOAs mediated by either parent styrene or non-CYP2F2 generated styrene metabolites (*e.g.*, styrene oxide) are unlikely.

2. Toxic Effects Are Limited to Lung Bronchiolar Epithelium in Mice Only

Toxic effects in the mouse lung from styrene have been demonstrated following inhalation, oral, or intraperitoneal (IP) administration. In studies with up to 2 weeks of exposure, increased cells and protein is found in broncho-alveolar lavage fluid (BALF), cell replication is increased (as measured by bromodeoxyuridine (BrdU) incorporation) in lung bronchiolar epithelium but not in alveolar cells, the intensity of staining of the endoplasmic reticulum in bronchiolar cells is diminished, and hyperplasia is observed in terminal bronchioles. Longer exposures indicate continued increased cell replication causing hyperplasia, which eventually extends into alveolar ducts.

No toxic effects are seen in alveolar cells at any time point from styrene exposure, even up to 2 years.

Using whole lung homogenates from C57BL/6 mice exposed to 40 or 120 ppm styrene by inhalation for 1 or 4 weeks (6 hrs/day for 5 days/week), full genomic evaluation indicated that styrene dramatically increases the expression of genes controlling cell cycle and replication.

In rat lungs, no cells are affected even at inhalation concentrations that are 8-fold higher – up to 1,000 ppm styrene 6 hrs/day 5 days/week – for 2 years. (Cruzan et al., 1998) In mice, Club (formerly called Clara) cells are affected. Increased BrdU labeling occurs only in bronchiolar tissue. Hyperplasia is found only in terminal bronchiolar tissues. Using enriched cell fractions from mouse lungs, Carlson reported that metabolism of styrene occurred only in Club (Clara) cells (Hynes et al., 1999).

Some have suggested that styrene is metabolized in Club cells and the metabolites cause events in nearby alveolar cells that lead to tumors. There is no evidence of toxicity or mutagenicity in alveolar cells in mouse lung. In fact using RNA from whole lung of C57BL/6 mice exposed to styrene at 40 or 160 ppm 5 days/week for 1 or 4 weeks, there was no indication of mutagenic events in the lung.

3. While Styrene Has Some Genotoxic Potential *In Vitro*, There Is No Convincing Evidence That Styrene Is Genotoxic *In Vivo*.

The overall picture presented by the available *in vitro* assay results available is that at least in some test systems (including tests from *in vitro* chromosome aberration studies in mammalian cells), styrene has some genotoxic potential *in vitro*. However, based on standard *in vivo* regulatory tests, arguably the more relevant testing environment, there is no convincing evidence that styrene is mutagenic/clastogenic.

In vitro mutagenicity assays (Ames) of styrene are negative (IARC, 1994). Micronucleus and chromosomal aberration assays in rats and mice are negative (IARC, 1994, 2002). Since styrene causes increased tumors only in mouse lung, assessment of genotoxicity in mouse lung is most relevant. Limited assays of genotoxic potential have been conducted in the lungs of mice exposed to styrene. There were no increases in chromosomal aberrations in the lungs of B6C3F1 mice exposed to 125, 250 or 500 ppm styrene for 2 weeks (Kligerman et al. 1993). Using A/J mice, a strain very susceptible to lung tumor formation, in an initiation/promotion assay, styrene administered for 7 weeks by IP injection did not initiate lung tumor formation (Brunnemann et al., 1992).

Other data are often cited to support a genotoxic MOA for styrene. *In vitro* mutagenicity assays of styrene-7,8-oxide (SO) are generally positive when epoxide hydrolase is inhibited (IARC, 1994). *In vivo* assays of micronucleus formation and chromosomal aberrations following exposure to SO were of mixed results, about half positive and half negative. However as described later, lung toxicity and tumor formation in mice from styrene exposure does not appear to be related to SO.

Studies in workers exposed to styrene and *in vitro* studies using human cells provide conflicting results. Most *in vitro* studies of micronucleus formation and chromosomal aberrations using human lymphocytes are positive (IARC, 1994, 2002). (This is in contrast to the *in vivo* studies in rats and mice, which are uniformly negative.) About 30 studies of workers in industries where workers are exposed to styrene have exhibited micronucleus and/or chromosomal aberrations. There does not seem to be any correlation between styrene exposure and micronucleus formation. However, about half of the chromosomal aberration studies are positive.

The one assay that seems to be consistently positive across *in vitro*, animal, and human studies is sister chromatid exchange (SCE). However, the Organisation for Economic Cooperation and Development (OECD 2014) recently removed SCE from the list of acceptable assays for genotoxicity because there is no functional connection between SCE and tumor formation.

4. DNA Adducts from Styrene Are Not Sufficient to Cause Tumors

Exposure to styrene by mice, rats and humans results in N-7-SO DNA adducts, as well as other adducts. Some authors have taken the findings of DNA adducts in mouse lung as an indication of a mutagenic MOA for styrene-induced mouse lung tumors and SO as the tumorigenic metabolite. Exposure of mice to 40 ppm styrene vapors for 2 years results in increased lung tumors; short-term exposures to this level of styrene in mice results in DNA adducts in lung. The lung DNA adducts are not an indication of a mutagenic MOA in lung since there are a greater number of adducts/gram tissue in liver, but no increase in liver tumors. *I.e.*, DNA adducts from styrene are not sufficient to cause tumors. Furthermore, rats exposed to 500 ppm styrene vapor develop more

DNA adducts in lung than do mice exposed to 40 ppm styrene, yet rats do not develop lung tumors. *I.e.*, DNA adducts from styrene are not sufficient to cause tumors.

The role of DNA adducts, in general, for causing mutations that lead to tumors as a linear, non-threshold MOA is questioned by recent research on aflatoxin. Johnson et al., (2014) recently published a set of experiments on the MOA of aflatoxin-induced liver cancer and chemoprevention. Aflatoxin B1 (AFB1) forms N-7-guanine adducts, produces GST-P-positive foci and eventually hepatocellular carcinoma. A specific gene signature is produced by AFB1 exposure. Administration of 200 μg/kg AFB1 via daily gavage for 4 weeks to F344 rats resulted in 100 pmol N-7AF-Guanine adducts/mg creatinine. Lifetime exposure to this dose resulted in a 96% incidence of hepatocellular carcinoma. Simultaneous exposure to 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) reduced the DNA adducts by 66% (*i.e.*, DNA adducts in the AFB1/CDDO-Im treated rats was 34 pmol N-7AF-Guanine adducts/mg creatinine). The genomic signature was also altered. The incidence of hepatocellular carcinoma was 0.

This study demonstrates that even for a genotoxic carcinogen such as aflatoxin, there is a threshold of DNA damage before cancer occurs.

A commentary by Drs. Olden and Vulimiri (2014) noted:

> They showed that AFB1 is a classic genotoxic substance in that it binds covalently to DNA and induces mutations. In fact, DNA adduct formation exhibits a characteristic linear dose–response curve over a wide range. But, further analysis demonstrated a threshold mode of action, with respect to internal dose of active metabolite and hepatocarcinogenesis. That is, there was substantial adduct formation and DNA damage without having any affect on development of hepatocellular carcinoma.

Although a genotoxicity MOA is the NTP's default assumption and limited genotoxicity data are found for styrene, there is no evidence that styrene induces mouse lung tumors through a genotoxic MOA, and these are the only tumors found in animals.

5. Metabolic Activation is Essential to Mouse Lung Toxicity

Metabolism by CYP2F2 is absolutely essential for toxic effects in mouse lung from styrene exposure. Although CYP2E1 readily metabolizes styrene to styrene-7,8-oxide (SO), elimination of CYP2E1 (CYP2E1-null mice) had no impact on styrene-induced lung toxicity. Styrene lung toxicity is reduced in CYP2E1-null mice.

Preliminary studies demonstrated that inhibition of CYP2F2 by 5-phenyl-1-pentyne reduced the lung toxicity of styrene (Green et al., 2001). More recent studies using CYP2F2-null mice demonstrated a complete loss of lung toxicity from styrene in the absence of CYP2F2

metabolism (Cruzan et al, 2012 and Carlson 2012). These studies also indicate that SO is not the toxic agent from styrene, because there was no toxicity from SO in the absence of further metabolism by CYP2F2 (Cruzan et al., 2012).

Genomics analysis demonstrated altered expression of genes related to control of cell cycle in the lungs of mice exposed to styrene. There was no altered expression in the lungs of CYP2F2-null mice (Cruzan et al., 2015).[5]

The fourth study that NTP did not have an opportunity to consider is Shen et al., (2014), which demonstrated that toxic naphthalene metabolites are generated in the lung by CYP2F2 and not CYP2A5, while in the nose it is CYP2A5, not CYP2F2, that generates toxic naphthalene metabolites.

MOA Conclusions

Metabolism of styrene or styrene oxide in the mouse lung by CYP2F2 is required for toxicity. In the absence of CYP2F2, there is no increase in cells, lactate dehydrogenase (LDH), or protein in broncho-alveolar lavage fluid (BALF), no increase in bromodeoxyuridine (BrdU) incorporation, and no differential expression of cell cycle genes in lung. Based on the lung tumor initiation assay, lung chromosomal aberration assay and lung genomic analysis, there is no indication of a genotoxic MOA. Using mice with the human CYP2F1 gene in place of the normal mouse CYP2F2 indicates that humans are incapable of producing sufficient metabolites to cause the lung effects seen in mice.

Mode of Action (MOA) research evidences that the mouse lung tumors are caused by mouse-specific metabolism by CYP2F2, which does not occur in humans. Thus, the animal data in Cruzan et al., (2001) are irrelevant to human cancer risk. Thus, the animal data are less than sufficient and does not support the listing of styrene under Proposition 65.

D. Inadequate Numbers of Human or Animal Studies Preclude Listing

Even if Cruzan et al., (2001) did support listing, it would be the only animal study in support of listing because the NCI (1979) study does not support listing as it did not show sufficient evidence of tumorigenicity in the study mice. As the California Health and Welfare Agency explained in promulgating § 23506, in determining whether to list a chemical, "[t]he Agency will look to determine whether the authoritative body relied upon animal or human data in an amount sufficient to satisfy the criteria." A single study does not satisfy this criterion. As such, there are insufficient animal data to support the listing of styrene under Proposition 65.

[5] Bolstering this conclusion is a SIRC-sponsored 4-week styrene inhalation lung toxicity study in KO, WT, and TG mice that supported the conclusion that CYP2F2 metabolism was a key event in the production of lung toxicity in mice following short-term exposure of inhaled styrene vapor. The final report from this study is provided as an attachment to these comments.

IV. Conclusion

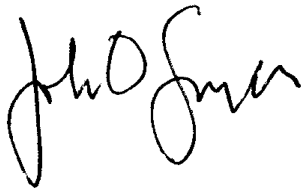
As detailed in these comments:

- Based on data that NTP did not consider, the human data do not support an association between exposure to styrene and human cancer, which precludes a listing.
- Based on data and scientific practices that NTP did not consider, the animal data are limited, not sufficient, which precludes a listing.
- Recent mode of action studies that NTP did not consider demonstrate that the mouse lung tumors identified in Cruzan et al., (2001) are not relevant to human cancer risk, which precludes a listing.

We recognize that OEHHA was obligated to consider an authoritative bodies listing for styrene based on a settlement agreement involving a number of chemicals. Based on the scientific record confronting OEHHA today, there is no basis for listing styrene under the authoritative bodies mechanism. Standing alone, the new human and mode of action studies that NTP did not consider preclude OEHHA from proceeding with an authoritative bodies listing. The impropriety of listing is further established by the absence of sufficient evidence of carcinogenicity in animal studies.

For these reasons, the NTP listing did not satisfy the sufficiency of evidence criteria to support an authoritative bodies listing and OEHHA should withdraw its Notice of Intent to List styrene.

Respectfully submitted,



John O. Snyder
Executive Director
Styrene Information & Research Center
910 17th Street NW – Suite 500B
Washington, DC 20006
Jack_Snyder@styrene.org / (202) 787-5996

Of Counsel:

Gene Livingston, Esq.
Greenberg Traurig
1201 K Street, Suite 1100
Sacramento, CA 95814-3938
Phone: (916) 442-1111

Of Counsel:

Peter L. de la Cruz, Esq.
Nathan A. Cardon, Esq.
Keller and Heckman, LLP
1001 G Street, N.W., Suite 500W
Washington, DC 20001
Phone: (202) 434-4100

REFERENCES

Brunnemann (1992) Brunnemann, K.D., A. Rivenson, S.C. Cheng, V. Saa, and D. Hoffmann. 1992. A study of tobacco carcinogenesis. XLVII. Bioassays of vinylpyridines for genotoxicity and for tumorigenicity in A/J mice. Cancer Lett. 65(2):107-113.

Carlson (2012) Carlson, G.P. 2012. Modification of the metabolism and toxicity of styrene and styrene oxide in hepatic cytochrome P450 reductase deficient mice and CYP2F2 deficient mice. Toxicology 294(2-3):104-108.

Collins et al., (2013) Collins J, Bodner K, and Bus S, Cancer Mortality of Workers Exposed to Styrene in the U.S. Reinforced Plastics and Composite Industry, Epidemiology 24:2, 195-203.

Coggon et al., (1987) Coggon D, Osmond C, Pannett B, Simmonds S, Winter PD, Acheson ED. 1987. Mortality of workers exposed to styrene in the manufacture of glass-reinforced plastics. Scand J Work Environ Health 13(2): 94-99.

Coggon et al., (2014) Coggon D, Ntani G, Harris E, Palmer K, Risk of cancer in workers exposed to styrene at eight British companies making glass reinforced plastics, Occup Environ Med doi:10.1136/oemed-2014-102382

Cruzan et al., (1998) Cruzan G, Cushman JR, Andrews LS, Granville GC, Johnson KA, Hardy CJ, Coombs DW, Mullins PA, Brown WR. 1998. Chronic toxicity/oncogenicity study of styrene in CD rats by inhalation exposure for 104 weeks. Toxicol Sci 46(2): 266-281.

Cruzan, et al., (2001) Cruzan G, Cushman JR, Andrews LS, Granville GC, Johnson KA, Bevan C, Hardy CJ, Coombs DW, Mullins PA, Brown WR. 2001. Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. J Appl Toxicol 21(3): 185-198.

Cruzan, et al., (2012) Cruzan, G; Bus, J; Hotchkiss, J; Harkema, J; Banton, M; Sarang, S. (2012). CYP2F2-generated metabolites, not styrene oxide, are a key event mediating the mode of action of styrene-induced mouse lung tumors. Regul Toxicol Pharmacol 62: 214-220. http://dx.doi.org/10.1016/j.yrtph.2011.10.007

Cruzan, et al., (2013) Cruzan, G; Bus, J; Hotchkiss, J; Sura, R; Moore, C; Yost, G; Banton, M; Sarang, S. (2013). Studies of styrene, styrene oxide and 4-hydroxystyrene toxicity in CYP2F2 knockout and CYP2F1 humanized mice support lack of human relevance for

mouse lung tumors. Regul Toxicol Pharmacol 66: 24-29. http://dx.doi.org/10.1016/j.yrtph.2013.02.008

Cruzan et al., (2015) Cruzan G, Bus J, Banton M, Sarang S, Dodd D, Black M, Andersen M. 2015. Mouse lung genomic responses in styrene treated wild-type, CYP2F2 knockout and CYP2F1 humanized mice support the low human relevance of mouse-specific lung toxicity and tumorigenicity. Tox. Sci. 144: 215

Green et al., (2001) Green, T., Toghill, A., Foster, J., 2001a. The role of cytochrome P-450 in styrene induced pulmonary toxicity and carcinogenicity in the mouse. Toxicology 169, 107–117.

Haseman et al., (1984) Haseman JK, Huff, J, Boorman, GA. 1984. Use of historical control data in carcinogenicity studies in rodents. Toxicologic Pathology 12: 126-135.

Haseman et al., (1985) Haseman JK, Huff JE, Rao GN, Arnold JE, Boorman GA, McConnell EE. 1985. Neoplasms observed in untreated and corn oil gavage control groups of F344 rats and (C57Bl/6N x C3H/HeN)F1 (B6C3F1) mice. JNCI. 75: 975-984.

Hynes et al., (1999) Hynes, DE, DiNicola, DB, Carlson, GP. 1999. Metabolism of styrene by mouse and rat isolated lung cells. Toxicol Sci. 51: 195-201.

IARC (2002) International Agency for Research on Cancer. 2002. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 82: 437-550 (Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene).

Keenan et al., (2009) Keenan C, Elmore S, Francke-Carroll S, Kemp R, Kerlin R, Peddada S, Pletcher J, Rinke M, Schmidt SP, Taylor I, and Wolf DC. 2009. Best Practices for Use of Historical Control Data of Proliferative Rodent Lesions. Toxicologic Pathology 37: 679-693.

Kligerman (1993) Kligerman AD, Allen JW, Erexson GL, Morgan DL. 1993. Cytogenetic studies of rodents exposed to styrene by inhalation. In Butadiene and Styrene: Assessment of Health Hazards, IARC Scientific Publications No. 127. Sorsa M, Peltonen K, Vainio H, Hemminki K, eds. Lyon, France: International Agency for Research on Cancer. p. 217-24.

Kogevinas (1994) Kogevinas M, Ferro G, Andersen A, Bellander T, Biocca M, Coggon D, Gennaro V, Hutchings S, Kolstad H, Lundberg I, Lynge E, Partanen T, Saracci R. 1994. Cancer mortality in a historical cohort study of workers exposed to styrene. Scand J Work Environ Health 20(4): 251-261.

Kolstad et al., (2014) 24th International Epidemiology in Occupational Health (EPICOH) Conference, "Challenges for Occupational Epidemiology in the 21st Century". 24-27 Jun 2014, Chicago, IL. Occup Environ Med 2014; v. 71, Suppl 1; pp A82-A83; doi:10.1136/oemed2014102362.257

NCI (1979) NCI. 1979a. Bioassay of Styrene for Possible Carcinogenicity. Technical Report Series No. 185. Bethesda, MD: National Cancer Institute.

OECD 2014] Organisation for Economic Cooperation and Development (OECD) "Following the OECD Council decision, the Test Guideline 479 'Genetic Toxicology: In vitro Sister Chromatid Exchange Assay in Mammalian Cells' was deleted on 2nd April 2014"; http://www.oecd-ilibrary.org/environment/test-no-479-genetic-toxicology-in-vitro-sister-chromatid-exchange-assay-in-mammalian-cells_9789264071384-en;jsessionid=3d3v1c79s4ku.x-oecd-live-03

Olden and Vulimiri (2014) Kenneth Olden K and Vulimiri s, Laboratory to Community: Chemoprevention Is the Answer, Cancer Prev Res July 1, 2014 7:648-652, 650.

Ruder et al., (2004) Ruder AM, Ward EM, Dong M, Okun AH, Davis-King K. 2004. Mortality patterns among workers exposed to styrene in the reinforced plastic boatbuilding industry: an update. Am J Ind Med 45(2): 165-176.

Ruder et al., (2014) 24th International Epidemiology in Occupational Health (EPICOH) Conference, "Challenges for Occupational Epidemiology in the 21st Century". 24-27 Jun 2014, Chicago, IL. The Ruder poster was published in Occup Environ Med 2014; v. 71, Suppl 1; pp A74-A75; doi:10.1136/oemed2014102362.232

Shen, et al., (2014) Shen, S; Li, L; Ding, X; Zheng, J. (2014). Metabolism of styrene to styrene oxide and vinylphenols in cytochrome P450 2F2- and P450 2E1-knockout mouse liver and lung microsomes. Chem Res Toxicol 27: 27-33. http://dx.doi.org/10.1021/tx400305w

Wong, et al., (1994) Wong O, Trent LS, Whorton MD. 1994. An updated cohort mortality study of workers exposed to styrene in the reinforced plastics and composites industry. Occup Environ Med 51(6): 386-396.